Incorporation of [^{3}H]glucosamine into keratin-related polypeptides in pig epidermis

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Metabolic labelling studies have provided evidence for glycosylated keratins in cultured pig epidermis. [^{3}H]Glucosamine was incorporated into five major particulate polypeptides of M_r 68 000, 61 000, 57 000, 53 000 and 48 000. Radioactivity was present in protein-bound carbohydrate. Non-enzymic glycation was excluded. Labelling was largely unaffected by tunicamycin indicating that radioactivity was incorporated mainly into *O*-linked oligosaccharides. These [^{3}H]glucosamine-labelled components were closely related to keratins since (i) they had a similar electrophoretic mobility to polypeptides of purified pig prekeratin, (ii) they were immunoprecipitated by anti-prekeratin serum and (iii) they were incorporated into reconstituted, intermediate-sized, keratin filaments.

Keratin polypeptide *Intermediate filament* *Glycoprotein synthesis* *Explant culture* *(Pig epidermis)*

1. INTRODUCTION

The keratins are a family of closely related polypeptides that are present as intermediate-sized filaments in the cytoskeleton of most, if not all, epithelial tissues. Although keratin filaments usually contain several polypeptides of M_r 70000–48000 their precise polypeptide composition shows remarkable variation within this size range [1–6]. This heterogeneity is due to differential gene expression [7] as well as post-translational modifications such as phosphorylation [8]. However, the possible glycosylation of keratin polypeptides has received little attention.

In previous studies of glycoprotein synthesis in the epidermis we [9,10] and others [11] have consistently observed incorporation of radioactive sugars into particulate components in the keratin M_r range. We now present evidence that these glycosylated polypeptides are closely related to keratins.

2. EXPERIMENTAL

2.1. *Preparation of prekeratin and anti-prekeratin serum*

Prekeratin was isolated from Dispase-separated pig ear epidermis [9] according to Matoltsy [1]. Rabbits received subcutaneous injections of prekeratin (2.5 mg) in complete Freund's adjuvant and were boosted with protein emulsified in incomplete adjuvant.

2.2. *Metabolic labelling*

Dispase-treated skin slices were labelled with D-[6-^{3}H]glucosamine hydrochloride (40 μCi/ml) or L-[4,5-^{3}H]leucine (20 μCi/ml) as described [9] and tunicamycin was used at 2 μg/ml [12]. Separated epidermis was homogenized, filtered through nylon mesh and the total particulate fraction prepared by centrifugation at 115000 $\times$ g_{av} for 1 h [10].

2.3. *Electrophoresis*

The Laemmli [13] procedure was used. Gels were stained with amido black and processed for fluorography [14].

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2.4. *Proteolytic digestion and acid hydrolysis*

Particulate fractions were run on a slab gel and the outer tracks were stained. The keratin region was excised from the rest of the gel (stored at −70°C), chopped and incubated with 1 mg/ml of pronase for 18 h at 37°C. The solubilized material was lyophilized, dissolved in 25 mM ammonium acetate, pH 6.8, applied to a column of BioGel P2 (1.5 × 85 cm) equilibrated in the same buffer and eluted at 20°C and 9 ml/h. Fractions of 3 ml were collected. Void volume fractions were lyophilized, hydrolysed in 4 M HCl at 100°C for 4 h and subjected to paper chromatography in ethyl acetate/pyridine/H_2O (10:4:3). 1 cm strips were eluted with H_2O before measurement of radioactivity.

2.5. *Immunoprecipitation*

Particulate fractions solubilized with 2% SDS at 100°C were adjusted to 0.1% SDS. 1% Nonidet P-40, 0.2 M NaCl, 1 mM PMSF, 1000 units/ml Trasylol, 50 mM Tris-HCl, pH 7.5 (immunoprecipitation buffer). 1 ml was incubated with 10 μl of antiserum for 4 h and 0.25 ml of protein A-bacterial absorbent (Miles Scientific) for 18 h. The absorbent was washed four times with immunoprecipitation buffer, once with H_2O and boiled in SDS sample buffer.

2.6. *Filament reconstitution*

Particulate fractions were extracted with 8 M urea, 1% 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5, for 1 h. After centrifugation at 35000 × g_{av} the extract was dialysed against 200 vols of 10 mM Tris-HCl, pH 7.5, for 18 h at 4°C. Aliquots were negatively stained with 1% uranyl acetate on carbon-formvar grids. Reconstituted filaments were collected by centrifugation at 35000 × g_{av}.

3. RESULTS

The polypeptide composition of prekeratin from pig ear epidermis (fig.1a) was similar to that of preparations from cow snout [4] and human epidermis [5]. It contained three major polypeptides (M_r 68000, 53000 and 48000) together with minor components (M_r 61000, 57000 and 51000). The polypeptides of M_r 68000, 61000, 57000, 53000 and 48000 were also the major components of the total particulate fraction from pig epidermis (fig.1b).

These five major particulate polypeptides were consistently labelled with [^{3}H]glucosamine (fig.2a) as well as [^{3}H]leucine in explant cultures (fig.2b). They were not labelled with [^{3}H]glucosamine in

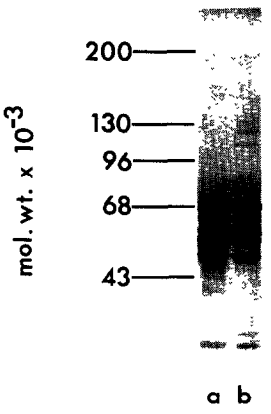

Fig.1. SDS gel electrophoresis of prekeratin (a) and total particulate fraction (b) isolated from pig epidermis.

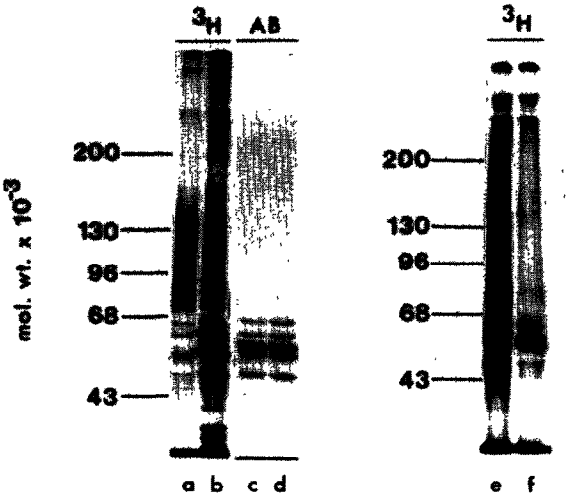

Fig.2. SDS gel electrophoresis of particulate fractions from metabolically labelled pig epidermis. The first panel compares the labelling of particulate components with [^{3}H]glucosamine (a) and [^{3}H]leucine (b). (c,d) Same tracks stained with amido black (AB). The second panel compares [^{3}H]glucosamine-labelling in untreated (c) and tunicamycin-treated epidermis (f).

tissue that had been heat treated (55°C, 5 min) or subjected to two cycles of freeze-thawing on solid CO_2. Their labelling is therefore unlikely to reflect non-enzymic glycation [17]. Tunicamycin which prevents the incorporation of [^{3}H]glucosamine into most membrane-bound glycoproteins in the epidermis [12] had little effect on the labelling of components in the keratin M_r range (fig.2f). Thus much of their radioactivity was present in *O*-glycosidically linked carbohydrates.

To confirm that radioactivity from [^{3}H]glucosamine was associated with protein-bound carbohydrate the region of M_r 68000–48000 from gels containing labelled particulate fractions was extensively digested with pronase and subjected to gel filtration. The elution of [^{3}H]glucosamine (fig.3a) was consistent with the presence of radioactivity in glycopeptides rather than peptides. Of the [^{3}H]glucosamine-labelled material, 85–90% was excluded by the gel while most of the [^{3}H]leucine-labelled peptides were retarded. Acid hydrolysis and paper chromatography of the [^{3}H]glucosamine-labelled material in the void volume showed that all of the radioactivity was recovered in hexosamine (fig.3b).

To determine whether the [^{3}H]glucosamine-labelled polypeptides of M_r 68000–48000 were immunologically related to keratins an antiserum was raised against pig prekeratin. The antiserum reacted predominantly with the prekeratin polypeptides of M_r 68000 and 53000 in immunoblotting (not shown). Similar reactivity of antiserum against cow prekeratin has been described [6]. In immunoprecipitation it specifically precipitated 4 or 5 of the major labelled polypeptides in the keratin M_r range. The reason for the

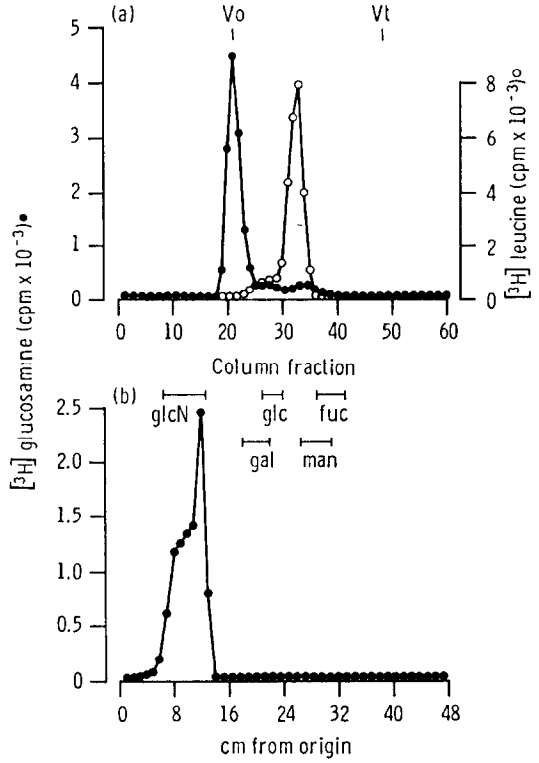

Fig.3. Gel filtration and paper chromatography of digests of the components of M_r 68000–48000 in particulate fractions of metabolically labelled epidermis. (a) Peptides released from the keratin region of SDS gels by pronase were fractionated on BioGel P2. Void volume (V_0) and total volume (V_t) were determined with ovalbumin and H_2O. (b) Sugars released from the [^{3}H]glucosamine-labelled material in the void volume by acid hydrolysis were separated by paper chromatography together with standard sugars.

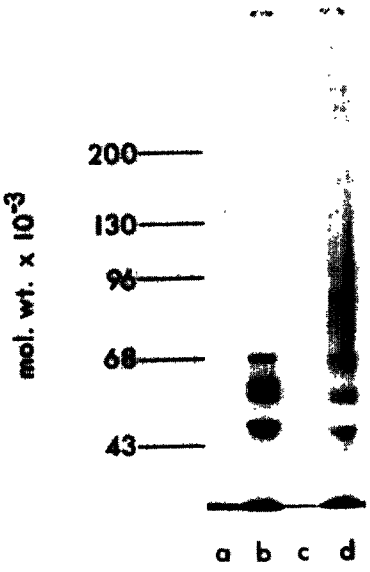

Fig.4. Immunoprecipitation of particulate fractions from [^{3}H]leucine (a,b) and [^{3}H]glucosamine-labelled (c,d) epidermis. Particulate fractions were solubilized with SDS, diluted into immunoprecipitation buffer and were incubated with preimmune rabbit serum (a,c) or anti-prekeratin serum (b,d) followed by protein A-bacterial absorbent.

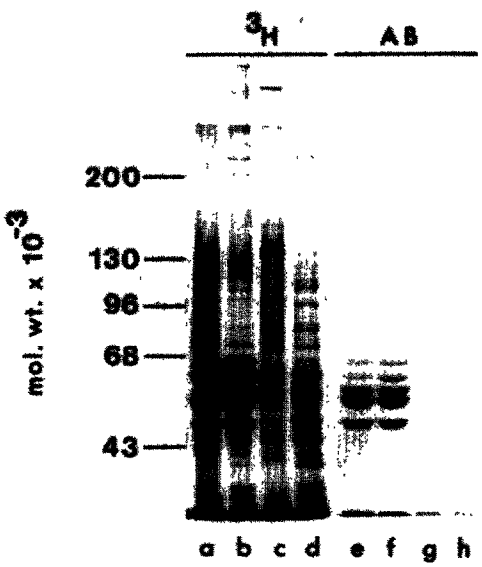

Fig.5. Reconstitution of intermediate-sized filaments from particulate fractions of [^{3}H]glucosamine (a,c) and [^{3}H]leucine-labelled (b,d) epidermis extracted with 8 M urea, 1% 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5. After dialysis against 10 mM Tris-HCl, pH 7.5, extracts were centrifuged at 35000 × g_{av} and one-tenth of the pelleted filaments (a,b) and supernatant fraction (c,d) were subjected to electrophoresis and fluorography (^{3}H). (e–h) Same tracks stained with amido black (AB).

variable number of polypeptides precipitated in individual experiments is not clear. However those polypeptides that were precipitated were always labelled with both [^{3}H]leucine (fig.4b) and [^{3}H]glucosamine (fig.4d).

To determine whether the [^{3}H]glucosamine-labelled polypeptides could be incorporated into intermediate-sized filaments, labelled particulate fractions were extracted with 8 M urea and 1% 2-mercaptoethanol. On removal of urea by dialysis, the major particulate proteins re-aggregated forming 8–10 nm filaments with a similar appearance in negative staining to the reconstituted filaments described by others [3,15]. On centrifugation of the dialysed extracts 64 ± 3 and 70 ± 2%, respectively, of the total [^{3}H]glucosamine and [^{3}H]leucine-labelled material was pelleted. The pelleted filaments were enriched in the 5 major [^{3}H]glucosamine-labelled polypeptides in the keratin M_r range (fig.5a) compared to the supernatant (fig.5c). The enrichment of [^{3}H]glucosamine-labelled polypeptides in reconstituted filaments was comparable with that found for [^{3}H]leucine-labelled (fig.5b,d) and amido black stained polypeptides (fig.5e–h).

4. DISCUSSION

Here we have shown that [^{3}H]glucosamine is incorporated into carbohydrate (probably mainly *O*-glycosidically linked) in polypeptides that have remarkably similar electrophoretic, immunological and solubility properties to polypeptides of prekeratin, a well characterized filamentous preparation derived from the living layers of the epidermis [1–3]. This would suggest that keratins occur in glycosylated forms at least in cultured tissue. It is not clear whether all newly synthesized keratins are glycosylated or whether a particular population is affected. The latter seems more likely in view of the considerable heterogeneity of keratin polypeptides at the cellular level [6]. Glycosylated keratins may be associated with a specific subpopulation of cells within the tissue or with particular subcellular elements of the cytoskeleton. In a preliminary report [16] it was suggested that keratin intermediate filaments in an epithelial carcinoma cell line were both glycosylated and exposed at the cell surface. However the [^{3}H]glucosamine-labelled polypeptides in the keratin M_r range in pig epidermis are unlikely to be cell surface-associated components since they were not susceptible to proteolytic degradation when intact epidermis was treated with trypsin (unpublished).

Whether glycosylation of keratins is a unique feature of cultured tissue is not yet clear. We have consistently observed concanavalin A-binding components of M_r 67000 and a doublet of M_r 52000 in detergent extracts of fresh epidermis and they were recognised by anti-prekeratin serum in immunoblotting (unpublished) suggesting that glycosylated keratins may also occur in vivo.

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